Interaction of Amide Inhibitors with the Active Site of Carbonic Anhydrase: Metal-Induced Deprotonation of the Bound Amide Group Is Indicated by Slow Binding Kinetics, by Visible Spectra of Complexes with Cobalt Enzyme, and by pH Effects on Binding Affinity†

Janice I. Rogers, Jogeshwar Mukherjee,‡ and Raja G. Khalifah*

Department of Biochemistry, Kansas University School of Medicine, and Veterans Administration Medical Center, Kansas City, Missouri 64128

Received January 14, 1987; Revised Manuscript Received May 11, 1987

ABSTRACT: Most carbonic anhydrase (CA) inhibitors bind at the active site metal and either are anions or are capable of deprotonation to yield anions. Much less is known about the interaction of CA with inhibitors that have hitherto been considered to bind as neutral species. We report a study of the reversible amide inhibition of Co(II)-substituted CA by iodoacetamide and ethyl carbamate (urethane), as well as the ambivalent oxamate, the monoamide of oxalate. Visible cobalt spectral changes indicate coordination of all these inhibitors to the metal. The pH dependence of the affinity of carbonic anydrase isozyme I (CA I) for ethyl carbamate and iodoacetamide is formally consistent with their binding either as anionic species to the acid form of the enzyme or as neutral species to the basic form of the enzyme. The former view is in better accord with the spectral data. Most strikingly, reversible binding of iodoacetamide and ethyl carbamate leads to uniquely slow kinetics of ligand association and dissociation that could be followed by simple mixing. The slow association kinetics suggest the involvement of energetically unfavorable deprotonation of the amide group preceding final coordination. The complex pH profile for inhibition of CA I by the ambivalent oxamate is consistent with coordination through the carboxylate group at low pH and through the deprotonated amide group at high pH. The visible spectrum of the complex of Co(II)CA I with oxamate shows a parallel dependence on pH, reflecting this dual coordination mode. Similarly, oxamate dissociation kinetics were biphasic and could be correlated with the pH-dependent spectral changes. Carbonic anhydrase thus appears to have a hitherto unrecognized high affinity for coordinating deprotonated amides at its active site metal.

The overwhelming majority of inhibitors of the zinc metalloenzyme carbonic anhydrase (EC 4.2.1.1) are known to bind by coordinating to the active site metal ion (Maren, 1967; Lindskog et al., 1971; Lindskog, 1982, 1983; Bertini & Luchinat, 1983). Such inhibitors are generally either monoanions or neutral molecules that are capable of deprotonation to yield anionic species. These inhibitors formally compete with hydroxide for binding to the active site metal ion and thus bind to the "acid" (zinc–water) form of the enzyme (Lindskog & Coleman, 1973; Bertini & Luchinat, 1983). This class includes the pharmacologically important sulfonamides that have been convincingly shown (Kanamori & Roberts, 1983; Blackburn et al., 1985) to bind to the active site metal through their deprotonated sulfonamido nitrogen. Bertini and Luchinat (1983) have given a lucid summary of the pH dependence for binding expected for these different cases.

The interaction of neutral inhibitors with carbonic anhydrase is also of considerable interest. Significantly, this small class includes the only two known competitive inhibitors of the physiological substrate CO_2, namely, imidazole (Khalifah, 1971, 1980) and phenol (Simonsson et al., 1982). It is noteworthy that the common hydration substrates of the enzyme, such as CO_2, aromatic esters, and aldehydes (Lindskog et al., 1971; Lindskog, 1982, 1983; Pocker & Sarkanen, 1978), are all neutral species not readily capable of ionization, and their mode of binding has not been elucidated. Similarly, many questions arise regarding the binding mode of the so-called neutral inhibitors (Jacob et al., 1980; Bertini & Luchinat, 1983; Simonsson et al., 1982; Tibell et al., 1985). This class also includes the weakly inhibitory alcohols and organic solvents (Verpoorte et al., 1967; Pocker & Stone, 1968), as well as amidic compounds such as ethyl carbamate (Whitney et al., 1967) and iodoacetamide (Whitney, 1970, 1973; Whitney et al., 1967). Many of these "neutral" inhibitors are capable, in principle, of a deprotonation at sufficiently alkaline pH, or their proton can be substituted by transition metal ions. In view of the high pK_a of amide groups (typically 14–15), it is perhaps not too surprising that they have been generally considered to bind only in their neutral form to the active site metal of CA.[1]

We now report that carbonic anhydrase has a hitherto unrecognized high affinity for coordinating amides and related compounds at its metal ion, a binding mode that involves the obligatory deprotonation of the amide group [cf. thorough review by Sigel and Martin (1982)]. The binding of amides as anions is thus strictly analogous to the interaction of the

† The financial support of the Medical Research Service of the Veterans Administration is gratefully acknowledged.

* Address correspondence to this author at Research Service (151), Veterans Administration Medical Center, 4801 Linwood Blvd., Kansas City, MO 64128.

‡ Present address: Department of Radiology, University of Chicago, Chicago, IL 60637.

[1] Abbreviations: CA, carbonic anhydrase; CA I and CA II, carbonic anhydrase isozymes I and II (formerly referred to as B and C type); CmCA I, CA I specifically carboxymethylated at its active site His-200; PAMBS, *p*-(aminomethyl)benzenesulfonamide; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

0006-2960/87/0426-5672$01.50/0

enzyme with the ionizable sulfonamides. This has been inferred from the following observations: (1) a characteristic pH dependence of the equilibrium constant for binding; (2) unique visible spectra of complexes with cobalt-substituted CA; (3) remarkably slow association and dissociation kinetics for formation of the enzyme–inhibitor complexes. Our study examined the *reversible* inhibition of CA by iodoacetamide, a covalent modifier of His-200 of CA isozyme I, as well as the inhibition by ethyl carbamate (urethane, an amide ester) and oxamate, the monoamide of oxalate. In view of the similarity in coordination properties between deprotonated amides and deprotonated "pyrrole" nitrogens of imidazoles (Sundberg & Martin, 1974; Sigel & Martin, 1982), our findings have bearing on the question (Bertini & Luchinat, 1983; Khalifah et al., 1987) of whether the imidazole anion is capable of binding to the active site metal of CA I.

Materials and Methods

Enzymes and Chemicals. Human carbonic anhydrase isozyme I was prepared from freshly outdated erythrocytes according to the affinity chromatography method developed earlier (Khalifah et al., 1977). Bovine carbonic anhydrase isozyme II was purchased from Sigma. Replacement of the intrinsic Zn(II) with Co(II) was carried out by first removing the Zn(II) with pyridine-2,6-dicarboxylate (Hunt et al., 1977), followed by stoichiometric addition of Specpure-grade cobalt sulfate (Johnson-Matthey). Cobalt CA I carboxymethylated specifically at His-200 [Co(II)CmCA I] was prepared by reacting Co(II)CA I with 15 mM bromoacetate in 0.1 M Bis-Tris buffer at pH 6.8 in the dark at room temperature for 6–7 h, followed by extensive dialysis. Protein concentrations were determined from the absorbance at 280 nm (Lindskog et al., 1971) or by the visible spectrum in the case of the Co(II) enzymes, using published extinction coefficients (Whitney, 1970; Taylor et al., 1970). Oxamate, iodoacetamide, and urethane were all from Sigma. Oxamate was also recrystallized and used in inhibitor binding studies to check for effects from possible oxalate contamination. (None were found.)

Spectrophotometry. All spectra were recorded on a Varian Cary 210 spectrophotometer equipped with a digital interface port (DIP) and interfaced to an Apple II+/IIe via a parallel interface card (SSM AIO). Software for acquisition, storage, and processing of spectra was developed in this laboratory and was written in Microsoft TASC-compiled BASIC language. Timing for kinetics runs was generated with a Mountain Hardware clock card for the Apple II+ computer, and data were automatically logged into the computer for analysis.

Inhibitor Binding Measurements. Inhibitor dissociation constants for the binding of various amides to the Co(II)-substituted enzymes were determined by visible spectral titrations. Complete spectra were recorded for each addition of inhibitor, and they were later corrected for dilution and, if necessary, for light scattering. The absorbance changes at a selected wavelength of maximal difference between native and inhibited enzyme, or the differences in absorbance at two chosen wavelengths, were analyzed as a function of inhibitor concentration with non-linear least-squares procedures assuming a simple binding equilibrium. Non-linear least-squares was also used to analyze the pH dependence of binding constants, as discussed in the text.

Computation of Limiting Spectra of Enzyme–Inhibitor Complexes. In order to obtain the limiting spectra of enzyme–inhibitor complexes at each pH, an extrapolation procedure was necessary, since complete saturation of the enzyme could not be achieved due to factors such as weak binding and limited solubility of the inhibitor. The following procedure was thus devised and implemented. By use of the "best" K_i determined at each pH by nonlinear least-squares fit of absorbance changes at wavelengths of maximal difference (greatest accuracy), a constrained least-squares analysis with the K_i fixed to that value was performed at all the other wavelengths in order to determine the extrapolated molar extinctions for the fully complexed enzyme. This was programmed for automated processing on the Apple II+/IIe.

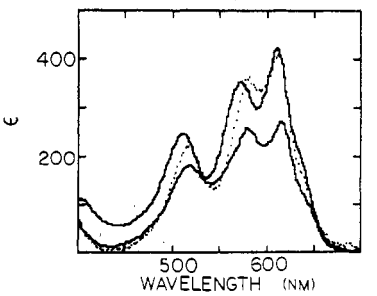


FIGURE 1: Computed (limiting) spectra of the ethyl carbamate complex of Co(II)CA I at pH 8.02 (upper solid curve), iodoacetamide complex of Co(II)CmCA I at pH 8.78 (lower solid curve), and iodoacetamide complex of Co(II)CA I at pH 8.00 (dotted curve).

Kinetics Measurements. Kinetics of association and dissociation were measured at a single wavelength chosen for maximal difference between initial and final spectra. Absorbances were obtained at specified time intervals and stored for kinetics analysis. All kinetics were done at room temperature (25 °C). An aliquot of the inhibitor was added in the cuvette at zero time for association kinetics. Two methods were used to initiate dissociation kinetics: (1) a 10-fold dilution of an equilibrated enzyme–inhibitor solution or (2) displacement of bound inhibitor by addition of an aliquot in a small volume of the extremely strong inhibitor to the equilibrated enzyme–inhibitor sample. A small plastic mixer was often used to carry out the addition and mixing. "Dead times" of this procedure were usually a few seconds. A nonlinear least-squares procedure was used to fit the data to a single exponential. Association kinetics were carried out under pseudo-first-order conditions of excess inhibitor.

Results

Reversible Binding of Iodoacetamide to Co(II)CA I. The *covalent* modification of CA I at His-200 by iodoacetamide has been thoroughly described by Whitney (Whitney et al., 1967; Whitney, 1970, 1973). He reported that the maximal half-time for inactivation is approximately 1–3 h for enzyme reversibly saturated with reagent (Whitney, 1970). In the present work, we studied the *reversible* binding by monitoring visible spectral changes in Co(II)CA I produced by initial binding of this reagent. Spectra were scanned shortly after addition of aliquots of inhibitor. Long incubation times at high iodoacetamide concentrations led to slow spectral changes that were due to a combination of covalent modification and the displacement of the reversibly bound iodoacetamide by the strongly inhibitory iodide produced by hydrolysis of the reagent at alkaline pH. Figure 1 (dotted curve) gives the computed limiting spectrum for the reversible complex of Co(II)CA I with iodoacetamide at pH 8.0. It differs entirely from the spectrum of the uninhibited enzyme [cf. Whitney (1970)]. The computed K_i value from the spectral titration at pH 8.0 was 40 ± 19 mM. This is in excellent agreement with Whitney's reported K_i of 25 mM at pH 7.6 for the zinc enzyme. On the basis of $[I]_{50}$ values, Whitney (1970, 1973) reported that the inhibition is pH independent at alkaline pH but falls off at low pH.

Table I: Kinetics of Inhibitor Binding to Co(II)CA at 25 °C[a]

reactants[b]	[I] (mM)	k_{obsd} (s^{-1})[c]	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_d/k_a (M)	K_i (M)[d]
Co(II)CA I, iodoacetamide, pH 8.1	6.0[e]	0.043				
	8.1	0.063				
	16.0	0.056				
	38.8	0.062				
	60.0	0.120				
	74.1	0.088				
			0.77 (±0.26)	0.041 (±0.008); 0.043 (±0.006)[f]	0.059 (±0.021)	0.040 (±0.020)
Co(II)CmCA I, iodoacetamide, pH 9.1	6.0[e]	0.147				
	6.0[e]	0.102				
	6.0[e]	0.139				
	60	0.163				
			0.65 (±0.365)	0.124 (±0.017)	0.20 (±0.11)	0.063 (±0.028)
Co(II)CA I, ethyl carbamate, pH 10.1	1.79	0.141				
	2.14[e]	0.136				
	2.14[e]	0.123				
	2.14[e]	0.116				
	3.59	0.201				
	3.59	0.277				
	3.59	0.215				
	7.17	0.242				
	7.17	0.260				
			26.5 (±5.6)	0.087 (±0.019); 0.16 (±0.02)[f]	0.0046 (±0.0010)	0.0067 (±0.0013)

[a] Data were obtained at ambient temperature (25 °C) and an ionic strength of 0.2 adjusted with K_2SO_4. All kinetics were obtained by spectroscopic measurements at suitably chosen wavelengths. [b] The final inhibitor concentrations are given in column 2 and were always greater than 10-fold the enzyme concentrations. [c] Observed first-order rate constant for either association or dilution dissociation. It was assumed to be equal to $k_a[I] + k_d$, and a linear regression fit of the data of column 3 yielded the rate constants given in columns 4 and 5. [d] Equilibrium inhibitor dissociation constant obtained by visible spectral titrations of enzyme with inhibitor. [e] Dissociation kinetics in which inhibitor concentration was diluted 10-fold to the value given in these entries. [f] Dissociation rate constant obtained by addition of excess acetazolamide. Values are the average of several experiments in which the acetazolamide concentration was varied over a 10- or 20-fold range to verify lack of dependence on its concentration.

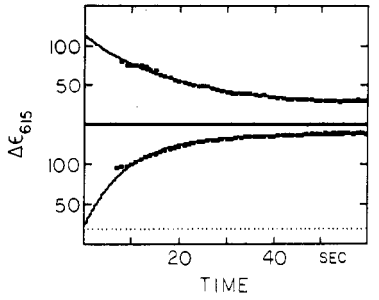


FIGURE 2: (Top) Dissociation kinetics for 10-fold dilution of 1.18 mM Co(II)CA I and 0.06 M iodoacetamide at pH 9.1. (Bottom) Kinetics of association of 0.023 M iodoacetamide with 0.18 mM Co(II)CA I. The dashed line represents the initial absorbance before addition of iodoacetamide. The curves represent nonlinear least-squares fit to a single-exponential function. The absorbance at 615 nm was followed in each case.

Kinetics of Binding of Iodoacetamide to Co(II)CA I. The kinetics of the visible spectral changes produced by the addition of iodoacetamide were possible to follow in a spectrophotometer by use of simple mixing, as shown in Figure 2, with half-times in the range of 10–15 s. The amplitude of the spectral change associated with the kinetics indicated that all the reaction was being observed. The apparent rate constant was linearly dependent on iodoacetamide concentration, as expected of a pseudo-first-order reaction. Similarly, the 10-fold dilution of a sample of Co(II)CA I to which iodoacetamide had been added produced spectral changes in the opposite direction (Figure 2). For an apparent reversible first-order reaction, the observed rate constant in *either* direction will be the sum of the dissociation rate constant and the pseudo-first-order association rate constant. We thus analyze the kinetics using $k_{obsd} = k_a[L] + k_d$, where the only restriction is that the ligand concentration is in large excess over the enzyme. The computed second-order association constants and the first-order dissociation constants are given in Table I. Dissociation could also be followed by the addition of the powerful inhibitor acetazolamide (diamox) to scavenge free enzyme, and the dissociation rate constant obtained in this way agreed with that measured by dilution (Table I). Furthermore, the k_d/k_a kinetic ratio was computed and found in accord with the equilibrium inhibitor dissociation constant obtained from analysis of the spectral titrations.

Reversible Binding of Iodoacetamide to Co(II)CmCA I. To further eliminate possible complications associated with covalent modification of His-200 by iodoacetamide, we studied the reversible binding of this inhibitor with enzyme already carboxymethylated at His-200. CmCA I has itself been found to reversibly bind iodoacetamide (Whitney, 1970). Figure 1 shows that the computed limiting spectrum for the reversible complex formed with this enzyme form is very similar to that of the complex with Co(II)CA I, except that it is somewhat less intense. The affinity of the modified enzyme for iodoacetamide appears weaker than for Co(II)CA I. The kinetics of spectral changes associated with addition of inhibitor or its dissociation by 10-fold dilution were also studied. The results are included in Table I. The weaker binding in the modified enzyme appears to reside in the larger value of the apparent dissociation rate constant.

Binding of Ethyl Carbamate. The addition of this inhibitor to Co(II)CA I produces large visible spectral changes indicating coordination to the metal. The limiting spectrum of the adduct with this inhibitor is shown in Figure 1. The K_i values for ethyl carbamate were determined by monitoring the visible spectral changes on addition of ethyl carbamate to Co(II)CA I over the pH range 6.0–10. Figure 3 shows that binding is considerably stronger at high pH, the profile being similar to that seen with ionizable inhibitors with high pK_a, such as the sulfonamides [cf. Bertini and Luchinat (1983)]. The pK_a obtained from the nonlinear least-squares fit of the data of Figure 3, 7.3 ± 0.2, is in excellent agreement with the free enzyme pK_a of 7.5, indicating that the active site ionization (Whitney, 1970) controls binding. A single K_i value of 5 mM at pH 7.6 has been reported for the inhibition of CA I by ethyl carbamate (Whitney et al., 1967), which is in full accord with our results.

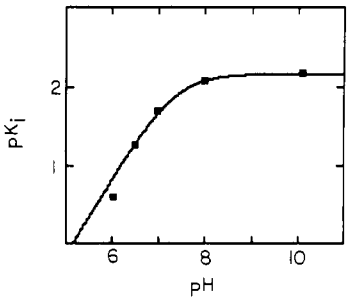


FIGURE 3: pH dependence of the ethyl carbamate dissociation constant of Co(II)CA I. The K_i values were obtained from visible spectral titrations. The solid curve represents a weighted three-parameter nonlinear regression fit to the experimental data in which binding was assumed to depend on a single ionization ($pK_a = 7.3 \pm 0.2$).

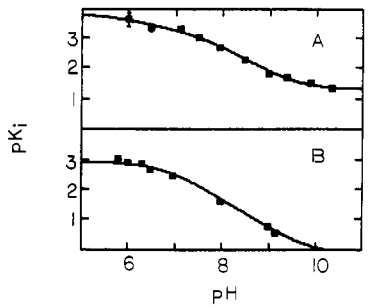


FIGURE 4: (A) pH dependence of the oxamate dissociation constant of Co(II)CA I. The K_i values were obtained from visible spectral titrations. The solid curve represents a weighted five-parameter nonlinear least-squares fit of the experimental data to eq 6 in which two enzyme ionizations were assumed to control binding, these being His-200 ($pK_a = 6.0$ assumed in free enzyme and 6.5 ± 0.7 found in complex) and zinc–water ($pK_a = 7.5 \pm 0.4$ found for free enzyme). The free ligand ionization was assumed to be 14 in the free ligand, and it was found to be 9.5 ± 0.2 in complex. (B) pH dependence of the oxamate dissociation constant of Co(II)CA II. The solid curve represents a weighted four-parameter nonlinear least-squares fit of the experimental data to eq 5. The free enzyme pK_a (zinc–water) was found to be 6.7 ± 0.2, and the ligand pK_a in the complex was found to be 9.8 ± 0.3. An equally satisfactory fit could also be obtained for a single ionization function such as in Figure 3.

Kinetics of Binding of Ethyl Carbamate to Co(II)CA I. As with the iodoacetamide inhibition, it was possible to follow the kinetics of ethyl carbamate association and dissociation through the visible spectral changes. However, in this case the rates were somewhat higher and were at the limit of our manual mixing methods. Analysis was facilitated by carrying out the nonlinear least-squares analysis while constraining the initial absorbance to the value observed before initiating the reaction. The association rate followed pseudo-first-order kinetics by being linearly dependent on the inhibitor concentration. Dissociation initiated by addition of the scavenging competing inhibitor acetazolamide gave a rate constant in reasonable agreement with that obtained by analysis of the dilution and association kinetics. Furthermore, the ratio of dissociation to association rate constants was in good agreement with the K_i measured by spectral titrations at equilibrium. The rate constants obtained are included in Table I.

Binding of Oxamate to Co(II)CA I. The K_i for oxamate inhibition of Co(II)CA I was determined by monitoring the visible spectral changes produced by the addition of oxamate. Figure 4A shows the pK_i values we obtained over the pH range 6–10. A complex dependence is seen. Below pH 9, the pH profile is approximately that expected for the typical binding of carboxylate and other anionic inhibitors to the acid form of the enzyme, with an observed controlling pK_a of 7.5 ± 0.4. However, at high pH, the binding affinity does not decrease logarithmically as expected. It tends to level off, suggesting

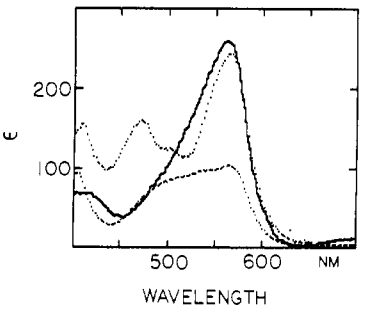


FIGURE 5: Computed (limiting) spectra of the oxamate complex of Co(II)CA I at pH 6.00 (lower dotted curve) and at pH 10.34 (solid curve) and of the oxamate complex of Co(II)CA II at pH 7.99 (upper dotted curve).

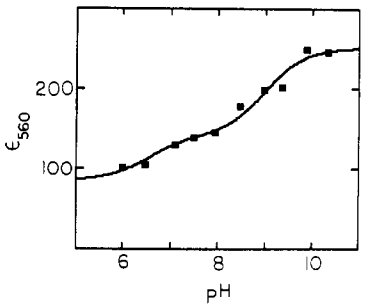


FIGURE 6: pH dependence of the 560-nm extinction coefficient of the limiting oxamate complex of Co(II)CA I. The solid curve represents a five-parameter nonlinear regression fit of the experimental data to a two pK_a function (constrained pK_a value of 6.5 and computed pK_a value of 9.0 ± 0.2).

that a deprotonation in this pH range leads to an increase in affinity. The curve drawn through the data will be discussed below.

Spectra of Limiting Complexes of Oxamate with Co(II)CA I. The limiting spectra of the adducts of oxamate with Co(II)CA I have been computed. The spectra appear to be highly pH dependent, as shown in Figure 5 for a high-pH and a low-pH case. It is interesting that the low-intensity spectrum at the acid extreme, but not the more intense one at high pH, is typical of the complexes of CA with carboxylate anions such as acetate or oxalate (Bertini et al., 1978a). A plot of the pH dependence of ϵ_{560nm} for the limiting complexes (Figure 6) reveals an apparent dependence on two ionizations. The curve drawn through the data represents a nonlinear least-squares fit to a dibasic acid function with apparent pK_a values of 6.5 (constrained) and 9.0 ± 0.2, as discussed below.

Kinetics of Inhibitor Dissociation of Oxamate. At high pH, weakened binding and limited solubility of the inhibitor made it difficult to observe association and dilution dissociation kinetics, such as the studies reported for ethyl carbamate and iodoacetamide. However, at high pH the dissociation was possible to observe by displacement with a high concentration of a strong inhibitor acting as a scavenger of free enzyme. This method was also advantageous, since the complex of Co(II)CA I with chosen displacing inhibitors, such as acetazolamide, has a very high extinction coefficient (Bertini et al., 1978b). Furthermore, sensitivity would be improved by addition of the acetazolamide in a small volume, thus avoiding dilution of the enzyme. Control experiments were carried out to establish that the measured rates were independent of the concentration of displacing inhibitors.

Table II summarizes the kinetics obtained by this method over the pH range of 8.2–10.4. Similar results were obtained by use of either acetazolamide or PAMBS as the displacing inhibitor. The first-order rate constant was largely independent of pH and was about an order of magnitude slower than the

Table II: Kinetics of Displacement of Oxamate from Co(II)CA at 25 °C[a]

enzyme	pH	k_d (s^{-1})	% fast[b]	displacing inhibitor
Co(II)CA I	8.22	0.018	93.4	6.9 mM diamox
	8.58	0.018	89.1	6.9 mM diamox
	8.98	0.035	70.6	6.9 mM diamox
	9.17	0.018	61.4	6.9 mM diamox
	9.38	0.021	49.5	6.9 mM diamox
	9.49	0.028	49.8	6.9 mM diamox
	9.50	0.014	29.0	86 mM diamox
	9.50	0.014	28.0	1.1 mM PAMBS
	9.50	0.015	31.0	1 mM PAMBS
	9.50	0.012	28.1	10 mM PAMBS
	9.50	0.021	34.1	10 mM PAMBS
	9.79	0.021	29.4	69 mM diamox
	9.98	0.020	29.0	69 mM diamox
	10.16	0.027	20.9	69 mM diamox
	10.39	0.037	13.4	69 mM diamox
Co(II)CA II	9.78	0.078	70.0	7.1 mM diamox

[a] Kinetics of dissociation of oxamate from its complex with Co(II)-CA isozymes I and II observed at 25 °C and ionic strength of 0.2 adjusted with K_2SO_4. Dissociation was induced by the addition of the competing inhibitors listed in column 5, and the kinetics were followed by the absorbance at 600 nm. [b] Three-parameter nonlinear least-squares (single exponential) was used to obtain the rate constants (column 3) as well as the initial and final absorbances. The computed spectral change was then compared to that expected from the initial absorbance before displacement, in order to arrive at the percent fast amplitude (column 4) that represented the unobservable fast reaction.

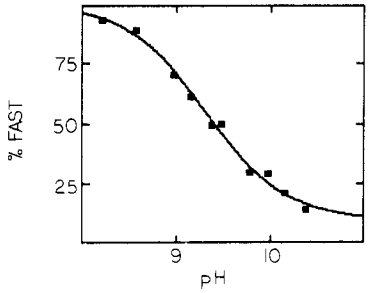


FIGURE 7: pH dependence of the percentage of 600-nm absorbance change that was too fast to observe during the dissociation of the oxamate complex with Co(II)CA I by diamox displacement. Note that the high- and low-pH complexes of oxamate (Figure 5) have a nearly equal absorbance at this wavelength that is much lower than that of the diamox complex. The solid curve represents a three-parameter nonlinear regression fit of the experimental data to a single ionization function (pK_a = 9.3 ± 0.2).

dissociation rates seen with iodoacetamide. The most remarkable aspect of these kinetics, however, was the pH dependence of the *amplitude* of the spectral change associated with the kinetics phase observed. Part of the spectral change occurred in the "dead time" of our slow mixing (a few seconds), so that the kinetics we measured did not extrapolate to the expected zero-time absorbance. Figure 7 shows a plot of the pH dependence of the percentage of the absorbance change associated with the dissociation reaction that was too fast to observe. The data were fit to a single ionization function, yielding a pK_a of 9.32 ± 0.06.

Binding of Oxamate to Co(II)CA II. The inhibition of Co(II)CA II by oxamate was also studied, and it was found to be weaker than that of Co(II)CA I. The spectrum of the limiting adduct (Figure 5) appeared to differ from that with Co(II)CA I in fine structure, and above pH 6.5 it did not appear to display the pH dependence that was strikingly seen with the other isozyme. The dependence of the pK_i on pH (Figure 4B) could not be followed above pH 9.0 due to weakened binding and limited inhibitor solubility. The pH dependence of the pK_i could be satisfactorily fit to a single ionization function, typical of binding as a simple anion to the acid form of the active site. A more complex function showing dependence on two ionizations could also be fit to the data (curve shown in Figure 4B). Displacement kinetics were attempted at pH near 10, and a slow kinetics phase (Table II) was observed corresponding to roughly 30 ± 5% of the total absorbance change.

Discussion

The visible spectra of the complexes of cobalt-substituted CA with the "neutral" inhibitors iodoacetamide and ethyl carbamate (Figure 1) and with the anionic oxamate (Figure 5) all indicate direct alterations in the first coordination sphere of the metal. Since nonionized amide nitrogens, as well as amide oxygens, are exceedingly poor ligands of metal ions (Sigel & Martin, 1982), the simplest interpretation of our spectra is to recognize that an amide proton is being substituted by the enzyme metal ion. As pointed out by Sigel and Martin (1982), the unfavorably high pK_a for amide deprotonation, typically near 15 (Stewart, 1985), can be compensated by the high ligand field strength of the resulting nitrogen anion, as well as by the possibility of chelate formation or other stabilizing interactions within the active site (Sigel & Martin, 1982).

The pH profiles for inhibition by ethyl carbamate (Figure 3) and iodoacetamide [cf. Figure 1 of Whitney (1970)] are fully consistent with the binding of the ligand anion (L) to only the acid form (EH) of the enzyme.[2] The ionizations involved are defined in eq 1 and 2, and the binding equilibrium is

$$EH = E + H \qquad K_a^E = [E][H]/[EH] \qquad (1)$$

$$LH = L + H \qquad K_a^L = [L][H]/[LH] \qquad (2)$$

defined in eq 3. The apparent inhibitor dissociation constant

$$EH + L = EHL \qquad K_L^{EH} = [EH][L]/[EHL] \qquad (3)$$

will then be given by the bell-shaped function of eq 4. A large

$$K_i = (1 + K_a^E/[H])(1 + [H]/K_a^L)K_L^{EH} \qquad (4)$$

separation between the two ionization constants leads to an appreciably flat plateau at pH values between the two pK_a's, and the upper or lower descending limbs of the plot will not be seen if the data cannot be extended toward the pH range of the corresponding pK_a values. Such is the case apparently with ethyl carbamate (Figure 3) and iodoacetamide [Figure 1 of Whitney (1970)]. The intrinsic inhibitor affinity, pK_L^{EH}, for binding of the fully deprotonated inhibitor to the fully protonated enzyme will exceed the apparent pK_i in the plateau region by *approximately* the difference $pK_a^L - pK_a^E$.

Agreement of the observed pH profiles with the predicted behavior from eq 4 cannot be taken as sufficient proof of the validity of this binding model, since it has been amply demonstrated that the identical type of pH dependence will result from a situation in which the acid form of the ligand (LH) binds to only the basic form (E) of the enzyme (Taylor et al., 1970). *Independent* evidence, such as the determination of the microscopic ionization state of ligand or enzyme, is needed to resolve the ambiguity. In the similar case of sulfonamide binding (Taylor & Burgen, 1971; Taylor et al., 1970), ^{15}N NMR spectroscopy (Kanamori & Roberts, 1983; Blackburn et al., 1985) showed the unambiguous presence of a singly protonated, i.e., ionized, sulfonamido nitrogen coordinated to

[2] Whitney (1970) attempted to explain the pH profile for iodoacetamide binding to CA I in terms of linkage to active site His-200. He made no reference to the catalytically essential ionizing group (zinc–water) known to control binding of anions and sulfonamides (Lindskog, 1982, 1983; Bertini & Luchinat, 1983).

the metal. As mentioned above, studies of model complexes of amides with metal ions clearly indicate that the likelihood of coordination of neutral amides to metal ions is vanishingly small (Sigel & Martin, 1982).

That amide deprotonation is required for binding of these neutral inhibitors also derives support from the kinetics studies that we have carried out. Observation of apparently slow ligand association and dissociation kinetics for binding to CA is the most remarkable and unexpected finding in our study. Whitney had noted the slow equilibration of iodoacetamide with CA I (Whitney et al., 1967; Whitney, 1970, 1973), but he did not report kinetics and he did not associate the slow equilibration, or the inhibition, with coordination at the metal ion site. There is no evidence to indicate that the slow kinetics are due to factors such as a conformational change in the enzyme. The kinetics we obtained with iodoacetamide for both mixing and dilution are clearly attributable to association and dissociation steps, since the ratio of the rate constants is in accord with the equilibrium binding constant. Furthermore, slow ligand binding kinetics have now been seen (Table I) for binding of iodoacetamide to modified Co(II)CmCA I, for binding of ethyl carbamate to Co(II)CA I, and for binding of oxamate (discussed below) to Co(II)CA I and Co(II)CA II, as well as for other amides (unpublished observations from this laboratory).

It is instructive to compare the association rate constants for binding of these amides with previously studied CA binding of other "unidentate" ligands, including the sulfonamides. Rate constants for association of the enzyme with the latter inhibitors approach the diffusion limit and usually exceed 10^6–10^9 M^{-1} s^{-1} (Taylor & Burgen, 1971; Taylor et al., 1970, 1971; Harrington & Wilkins, 1980).[3] Our observed k_a's in the range of 1–10 M^{-1} s^{-1} are thus truly remarkable. Slow dissociation rate constants of the order of 0.05 s^{-1} have been reported with sulfonamides (Lindskog & Thorslund, 1968; Taylor et al., 1970), and these usually arise from the extremely high affinity coupled with diffusion-controlled association ($k_d = k_a K_i$). The estimated *intrinsic* inhibitor dissociation constants for binding of the deprotonated amides to the protonated enzyme do indeed approach the nanomolar range. The extremely high affinity of the enzyme for deprotonated amides may reflect the excellent coordinating properties (basicity) of deprotonated amide (Siegel & Martin, 1982). Unlike the sulfonamides, the amides discussed here are small and lack aromatic or heterocyclic hydrophobic groups, making the comparison even more remarkable.

The basis for the slow association rate constant for binding of iodoacetamide to CA most likely arises from the obligatory deprotonation of the amide group prior to coordination to the active site metal. The maximum rate of spontaneous deprotonation of an ionizing group with pK_a of 15 will be about 10^{-6} s^{-1}, on the basis of a diffusion-limited protonation rate of about 10^9 M^{-1} s^{-1}. It is thus highly unfavorable from an energetic point of view. Deprotonation in free amides in alkaline solutions will proceed, of course, through hydrolysis rather than simple proton dissociation (Eigen, 1964). However, if deprotonation occurs following an initially formed complex of amide with the metal, access of hydroxyls could be quite hindered. Much more searching kinetics studies would be needed to elucidate the reaction, and the possible involvement of slow proton-transfer reactions makes the study of solvent deuterium isotope effects attractive. Such studies are in progress.

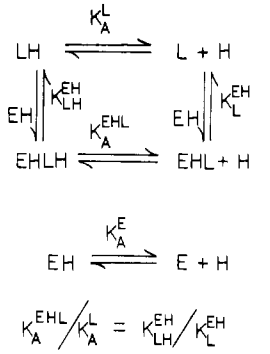


FIGURE 8: Inhibitor binding model in which only the acid form of the enzyme (EH) is assumed to bind to the protonated (LH) and the deprotonated (L) forms of the ligand. Note that pK_a^{EHL} represents the ionization of the ligand within the enzyme–ligand complex.

If our interpretation of the basis for the slow association rate of iodoacetamide is correct, slow binding kinetics should be characteristic of inhibition of CA by most amides. The oxamate results confirm this, although the inhibition of Co(II)CA I by oxamate presents interesting complications. This potentially bifunctional and bidentate ligand binds at low pH to the metal as the monoanion through its carboxylate group, as judged by the low-intensity visible spectrum of its complex (Figure 5) and by the decrease in its affinity for the enzyme up to about pH 9 (Figure 4A). At higher pH, the large spectral change of its complex clearly indicates alterations in its mode of coordination with the metal. This is strongly suggestive of a change to binding of the dianion through its deprotonated amide group (with obvious possibilities for chelation in a five-membered ring).[4] It is possible to quantitatively correlate the pH dependence of binding and the independently obtained pH dependence of the spectral changes if we assume that the oxamate anion (LH) and the dianion (L) both bind to the acid form of the enzyme (EH), as shown in Figure 8. The apparent inhibitor dissociation constant for this situation is given by eq 5. We have chosen to express

$$K_i = \frac{(K_a^L K_{LH}^{EH}/K_a^{EHL})(1 + [H]/K_a^E)(1 + [H]/K_a^L)}{([H]/K_a^E)(1 + [H]/K_a^{EHL})} \quad (5)$$

the apparent pK_i in terms of the enzyme and ligand ionizations, K_a^E and K_a^L, respectively, and the ionization constant of the enzyme–inhibitor complex (K_a^{EHL}). The latter represents the pK_a of the pH-linked change from coordination of the oxamate anion through the carboxylate (EHLH species) to coordination of the dianion through the deprotonated amide group (EHL). If these two complexes have different visible spectra, then a spectral change with this pK_a is naturally predicted by this model, and eq 5 indicates that the pK_a for the change can be *independently* obtained by analysis of the binding data. A nonlinear least-squares fit of the data for Co(II)CA I inhibition (Figure 4A) yielded a pK_a^{EHL} of 9.4 ± 0.2. The spectral changes of Figure 6 do indeed reveal a transition in this neighborhood and thus support the self-consistency of this analysis of the binding and spectral data.

An additional spectral change also occurs, however, at lower pH with an inflection near 6.5. Such changes have been seen before in Co(II)CA I and Co(II)CmCA I (Whitney, 1970), as well as in some of their inhibitor complexes (Whitney &

[3] An exception is the binding of the bidentate ligand pyridine-2,6-dicarboxylate, which rapidly extracts the metal ion from CA (Hunt et al., 1977). Its association rate constant is in the range of 10^2 to 10^3 M^{-1} s^{-1} (Harrington & Wilkins, 1980).

[4] It is interesting in this regard that Bertini et al. (1978a) found that oxalate binding to CA is strongest among dicarboxylates.

Brandt, 1976), and they are assigned to pertubation by the adjacent active site His-200 (Khalifah, 1977). In the case of oxamate, the observed pK_a of 6.5 for this residue in the *complex* differs from that (6.0) in the free enzyme, indicating that its effect *must* be reflected in the pH dependence of binding [cf. discussion of linkage in Khalifah (1977)]. The curve in Figure 4A is a nonlinear least-squares fit to the scheme of Figure 8 to which we added linkage to this second enzyme group (His-200), resulting in the following pH dependence:

$$K_i = \frac{K_{a1}^{E} K_a^{L} K_{LH}^{HEH}(1 + [H]/K_a^{L})[1 + ([H]/K_{a1}^{E})(1 + [H]/K_{a2}^{E})]}{K_{a1}^{EL} K_{a2}^{EL}([H]/K_{a2}^{E})[1 + ([H]/K_{a1}^{EL})(1 + [H]/K_{a2}^{EL})]} \quad (6)$$

This analysis of the data of Figure 4A yielded a pK_{a1}^{EL} of 6.5 ± 0.7 for His-200 in the complex and a pK_{a2}^{EL} of 9.4 ± 0.2 for the ionization governing the change from coordination through the carboxylate to coordination through the amide. These data are thus in full accord with the spectral changes seen in Figure 6.

In considering the analysis of the full pH dependence of binding discussed above, it is important to recognize that the alternate view, in which the neutral amide group of oxamate binds at high pH to the basic form of enzyme, leads to an identical dependence of binding on pH and can thus *formally* account for the pH dependence of binding. In that case, however, the observed spectral change of pK_a = 9.4 would have to be assigned to an *enzyme* ionization within the complex. There is no independent evidence supporting the existence of such an ionization in the active site. As in the case of the simple amides, a final distinction between these models can only rest on a direct demonstration of the state of ionization of the amide group in the complex.

The conclusion that binding of oxamate to Co(II)CA I should occur through the deprotonated amide group as the pH is raised provides an opportunity to test our earlier prediction that slow kinetics should occur with amide binding. We consequently searched for and successfully measured slow dissociation kinetics at alkaline pH (Table II). Not only were slow kinetics observed, but the amplitude of the changes (Figure 7) precisely mirrored the fraction of enzyme–inhibitor complex computed from the spectral and binding equilibrium to be coordinated through the deprotonated amide group. It is noteworthy that the dissociation rate constant in the alkaline range is essentially pH independent (Table II), which is also the case for dissociation of sulfonamides from CA (Taylor et al., 1970).

Exploratory kinetics studies on the oxamate inhibition of Co(II)CA II were also carried out. The weaker inhibition of this isozyme (Figure 4B) made it difficult to observe the expected change of coordination from binding through the carboxylate to binding through the anion. Nonlinear least-squares analysis of the binding data (Figure 4B) suggested that the transition occurs with a pK_a of 9.8 ± 0.3. A slowly dissociating phase of about 30 ± 5% amplitude was observed near pH 10, indicating that this isozyme can also coordinate amides. However, computation of limiting spectra of the complex with enzyme could not be carried out at such high pH due to weak binding and poor inhibitor solubility.

To summarize, the binding of amide inhibitors of carbonic anhydrase appears to occur by substitution of an amide proton by the metal ion of the active site. Visible spectra, pH dependence of the binding, and characteristically slow association–dissociation kinetics all implicate binding of such inhibitors following their deprotonation. It would be especially difficult to account for the spectral changes and the slow kinetics in terms of the alternate view in which neutral amides coordinate to the acid form of the enzyme. The interaction of amides and related compounds must now be considered strictly analogous to the binding of sulfonamides and anionic inhibitors, and such binding occurs competitively with solvent hydroxyls for binding at the metal. A definitive proof of such an interaction is not available at present, and it must be sought by spectroscopic and other methods capable of directly observing the ionization state of the inhibitor on the enzyme.

Binding of amides in their deprotonated form may help account for observations on relative inhibitor potency. Whitney et al. (1967) have previously reported that inhibition by acetamide and urea is weaker than that of iodoacetamide and chloroacetamide by a little less than 2 orders of magnitude. The amide groups of the latter compounds are, indeed, expected to be more acidic than the former by about 2 pH units (Stewart, 1985). The binding of deprotonated amides to carbonic anhydrase also provides an explanation of why the enzyme does not catalyze their hydrolysis. This mode of coordination is well-known [cf. Sigel and Martin (1982)] to make the amide group exceedingly stable to hydrolysis.

The similarity of coordinating properties and pK_a's between amides and "pyrrole" nitrogens of imidazoles has been noted (Sundberg & Martin, 1974; Sigel & Martin, 1982). Our present results may thus be relevant for understanding the mode of binding of the CO_2 competitive inhibitor imidazole (Khalifah, 1971). Hitherto, imidazole and related analogues have been considered to bind only in their neutral form through their "pyridine" nitrogen, without displacing the coordinated water ligand of the metal (Kannan et al., 1977; Wolpert et al., 1977; Bauer et al., 1977; Alberti et al., 1981). An alternative view of their binding has been noted (Bertini & Luchinat, 1983) in which imidazole binds as the neutral species to the acid form of the enzyme at low pH, but at high pH it binds as the anion and coordinates to the metal through its deprotonated "pyrrole" nitrogen. This ambivalent mode of binding should lead to a similar pH dependence of the affinity to that seen with oxamate and Co(II)CA I in our present study [cf. Khalifah et al. (1987)].

In conclusion, we believe our studies on the inhibition of CA by neutral inhibitors are fully consistent with many observations on model complexes. *Neutral inhibitors capable of deprotonation bind at the metal only through their anionic form when only the anionic forms are potentially excellent ligands of metal ions.* Claims to the contrary need to be critically examined.

References

Alberti, G., Bertini, I., Luchinat, C., & Scozzafava, A. (1981) *Biochim. Biophys. Acta 668*, 16–26.

Bauer, R., Limkilde, P., & Johansen, J. (1977) *Carlsberg Res. Commun. 42*, 325–339.

Bertini, I., & Luchinat, C. (1983) *Acc. Chem. Res. 16*, 272–279.

Bertini, I., Luchinat, C., & Scozzafava, A. (1978a) *Bioinorg. Chem. 9*, 93–100.

Bertini, I., Canti, G., Luchinat, C., & Sozzafava, A. (1978b) *J. Am. Chem. Soc. 10*, 4873–4877.

Blackburn, G. M., Mann, B. E., Taylor, B. F., & Worrall, A. F. (1985) *Eur. J. Biochem. 153*, 553–558.

Eigen, M. (1967) *Angew. Chem., Int. Ed. Engl. 3*, 1–72.

Harrington, P. C., & Wilkins, R. G. (1980) *J. Inorg. Biochem. 12*, 107–118.

Hunt, J. B., Rhee, M., & Storm, C. B. (1977) *Anal. Biochem. 79*, 614–617.

Jacob, G. S., Brown, R. D., & Koenig, S. H. (1980) *Biochemistry 19*, 3754–3765.
Kanamori, K., & Roberts, J. D. (1983) *Biochemistry 22*, 2658–2664.
Kannan, K. K., Petef, M., Fridborg, H., Cid-Dresdner, H., & Lovgren, S. (1977) *FEBS Lett. 73*, 115–119.
Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.
Khalifah, R. G. (1977) *Biochemistry 16*, 2236–2240.
Khalifah, R. G. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 206–215, Springer-Verlag, Berlin.
Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.
Khalifah, R. G., Rogers, J. I., & Mukherjee, J. (1987) *Biochemistry* (in press).
Lindskog, S. (1982) *Adv. Inorg. Biochem. 4*, 115–170.
Lindskog, S. (1983) in *Zinc Enzymes, Metal Ions in Biology* (Spiro, T. G., Ed.) pp 78–121, Wiley-Interscience, New York.
Lindskog, S., & Thorslund, A. (1968) *Eur. J. Biochem. 3*, 453–460.
Lindskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2505–2508.
Lindskog, S. Henderson, L E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes (3rd Ed.) 5*, 587–665.
Maren, T. H. (1967) *Physiol. Rev. 47*, 595–781.
Pocker, Y., & Stone, J. T. (1968) *Biochemistry 7*, 2936–2945.
Pocker, Y., & Sarkanen, S. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 149–274.
Sigel, H., & Martin, R. B. (1982) *Chem. Rev. 82*, 385–426.
Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1982) *Biochem. Biophys. Res. Commun. 108*, 1406–1412.
Stewart, R. (1985) in *The Proton: Applications to Organic Chemistry*, Academic, New York.
Sundberg, R. J., & Martin, R. B. (1974) *Chem. Rev. 74*, 471–517.
Taylor, P. W., & Burgen, A. S. V. (1971) *Biochemistry 10*, 3859–3866.
Taylor, P. W., King, R. W., & Burgen, A. S. V. (1970) *Biochemistry 9*, 3894–3902.
Taylor, P. W., Feeney, J., & Burgen, A. S. V. (1971) *Biochemistry 10*, 3866–3875.
Tibell, L., Forsman, C., Simonsson, I., & Lindskog, S. (1985) *Biochim. Biophys. Acta 829*, 202–208.
Verpoorte, J. A., Mehta, S., & Edsall, J. T. (1967) *J. Biol. Chem. 242*, 4221–4229.
Whitney, P. L. (1970) *Eur. J. Biochem. 16*, 126–135.
Whitney, P. L. (1973) *J. Biol. Chem. 248*, 2785–2789.
Whitney, P. L., & Brandt, H. (1976) *J. Biol. Chem. 251*, 3862–3867.
Whitney, P. L., Folsch, G., Nyman, P. O., & Malmstrom, B. G. (1967) *J. Biol. Chem. 242*, 4206–4211.
Wolpert, H. R., Strader, C. D., & Khalifah, R. G. (1977) *Biochemistry 16*, 5717–5721.

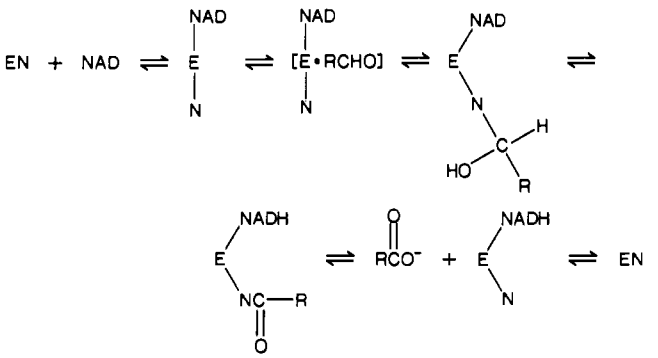
Active Site of Human Liver Aldehyde Dehydrogenase†

Darryl P. Abriola, Robert Fields, Stanley Stein, Alexander D. MacKerell, Jr., and Regina Pietruszko*
Center of Alcohol Studies, Busch Campus, Rutgers University, and New Jersey Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey 08854

Received March 4, 1987; Revised Manuscript Received April 16, 1987

ABSTRACT: Bromoacetophenone (2-bromo-1-phenylethanone) functions as an affinity reagent for human aldehyde dehydrogenase (EC 1.2.1.3) and has been found specifically to label a unique tryptic peptide in the enzyme. Amino-terminal sequence analysis of the labeled peptide after purification by two different procedures revealed the following sequence: Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys. Radioactivity was found to be associated with the glutamate residue, which was identified as Glu-268 by reference to the known amino acid sequence. This paper constitutes the first identification of an active site of aldehyde dehydrogenase.

Aldehyde dehydrogenase (EC 1.2.1.3) is the enzyme involved in metabolism of administered ethanol. It is a tetramer of M_r 216 000, which occurs in two isozymes (both homotetramers, one mitochondrial and the other cytoplasmic). It has broad substrate specificity and catalyzes an irreversible conversion of aldehydes to acids, employing NAD as coenzyme. The chemical steps, reaction intermediates, and catalytic groups utilized by aldehyde dehydrogenase in the oxidation of aldehydes remain virtually unknown but are believed to closely resemble those determined for glyceraldehyde-3-phosphate dehydrogenase. Jacoby (1963) and Weiner (1979) have proposed the general scheme:



In this scheme, the carbonyl carbon of the aldehyde is attacked by the enzyme (E) nucleophile (N), which by analogy with glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters,

† Financially supported by NIAAA Grant AA00186, Research Scientist Development Award AA00046, and the Charles and Johanna Busch Memorial Fund.
* Address correspondence to this author at the Center of Alcohol Studies.

0006-2960/87/0426-5679$01.50/0